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Loss of a Class A Penicillin-Binding Protein Alters β-Lactam Susceptibilities in *Mycobacterium tuberculosis*

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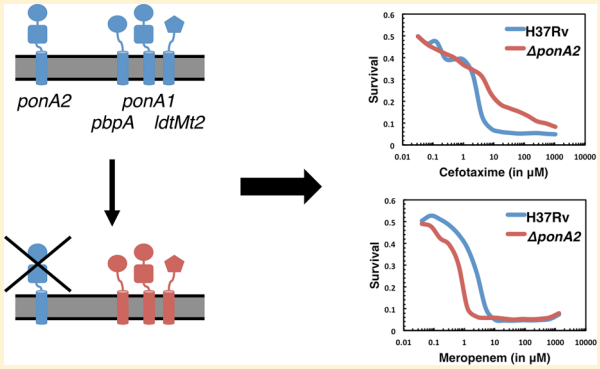
Supporting Information

ABSTRACT: Recent studies have renewed interest in β-lactam antibiotics as a potential treatment for *Mycobacterium tuberculosis* infection. To explore the opportunities and limitations of this approach, we sought to better understand potential resistance mechanisms to β-lactam antibiotics in *M. tuberculosis*. We identified mutations in the penicillin-binding protein (PBP) *ponA2* that were able to confer some degree of resistance to the cephalosporin subclass of β-lactams. Surprisingly, deletion of *ponA2* also confers resistance, demonstrating that β-lactam resistance can spontaneously arise from PBP loss of function. We show that *ponA2* mutants resistant to the cephalosporin subclass of β-lactams in fact show increased susceptibility to meropenem, a carbapenem that is known to target L,D-transpeptidases, thereby suggesting that in the absence of PonA2, an alternative mode of peptidoglycan synthesis likely becomes essential. Consistent with this hypothesis, a negative genetic selection identified the L,D-transpeptidase *ldtMt2* as essential in the absence of *ponA2*. The mechanism of β-lactam resistance we outline is consistent with emerging models of β-lactam killing, while the investigation of *ponA2* downstream and synthetic lethal genes sheds light on the mechanism of cell wall biosynthesis and the interaction between conventional PBPs and L,D-transpeptidases.



KEYWORDS: *actinobacteria, carbapenem, cephalosporin, loss of function, PonA2, target site modification*

Tuberculosis is a global threat that causes 1.4 million deaths per year.[1] In recent reports, the causative organism, *Mycobacterium tuberculosis* (MTB), has developed resistance to every first- and second-line antibiotic, evolving into a totally drug-resistant form.[2] At the same time, more common multidrug resistance to the two first-line agents, isoniazid and rifampin, is increasing in prevalence.[1] This increasing rate of resistance has stimulated recent interest in applying the β-lactam class of antibiotics to tuberculosis, given their historical success against other bacterial pathogens. The β-lactam class of antibiotics is rarely used in the clinic because of MTB's high intrinsic tolerance to β-lactams.[3] However, since the initial observation that meropenem effectively kills multidrug-resistant MTB,[4] recent work has shown that combination therapy with the β-lactamase inhibitor clavulanate may make several β-lactams viable treatment options.[5,6]

It has long been known that the β-lactams act by forming a long-lived acyl-enzyme intermediate at the catalytic serine of their target penicillin-binding proteins (PBPs). In this way, they inhibit the PBP's essential activity, leading to a decrease in bacterial viability.[7] The PBPs perform the essential activity of synthesizing the peptidoglycan sacculus, which encases the bacterium and helps to contain its turgor pressure.[8] They perform the final step of peptidoglycan synthesis, the cross-linking of peptide side chains on long glycan strands, thereby attaching newly synthesized glycan strands to the saccular macromolecule.

Despite a detailed understanding of the individual molecular components of the peptidoglycan synthesis pathway, the precise steps leading from PBP inhibition to cell death are poorly understood. The predominant model is that the PBPs perform a synthetic function that is in balance with peptidoglycan hydrolases. Inhibition of the synthetic function results in an unbalanced hydrolytic function that degrades the sacculus until it can no longer withstand the cellular turgor pressure, leading to release of the cellular contents and death.[8−11] Although this simple model is consistent with many observations concerning β-lactam-mediated cell death, β-lactam potency and the morphological alterations preceding death vary by β-lactam subclass.[12] This variation probably

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results from the differing affinities of different PBP paralogs for particular β-lactam structures. Further complicating the model is the existence of alternative transpeptidases known as L,D-transpeptidases, which act on a different part of the peptide cross-bridge than the PBPs. The presence of these transpeptidases can markedly alter β-lactam susceptibility.[13−16]

In this work, we sought to elucidate the β-lactam mechanism of action in the particular case of the cephalosporin class of β-lactams in *M. tuberculosis*. We found that mutations in the PBP *ponA2* confer resistance to cephalosporins; however, these mutations are recessive and result in loss of function of the *ponA2* transpeptidase domain, thereby suggesting that the classic alteration of the binding site affinity for the β-lactam is not the mechanism by which these mutations confer resistance. We further show that loss of *ponA2* transpeptidase function, in contrast to cephalosporins, confers sensitivity rather than resistance to a second class of β-lactams, the carbapenems. We reconcile these conflicting susceptibitilities by proposing that during cephalosporin treatment, the inhibition of cephalosporin-sensitive PBPs allows a toxic effect to result from the portion of peptidoglycan synthesis performed by PonA2. Meanwhile, during meropenem treatment, PonA2 acts as a resistance-determining PBP. In the absence of PonA2, an alternative, meropenem-sensitive transpeptidase is operating. We identify LdtMt2 as the most likely candidate for this alternative transpeptidase, given its known role as a target of meropenem.

RESULTS AND DISCUSSION

To expand on the growing body of work exploring the possibility of using β-lactams in MTB therapy, we wished to identify MTB's cephalosporin resistance-determining PBP. Generation of de novo resistant mutants to β-lactams in MTB has been challenging due to their intrinsic high MICs. Therefore, we took advantage of our previous finding that an MTB mutant strain, H37RvΔ*rpfACBED* (hereafter, H37RvΔ*rpf*), in which five peptidoglycan lytic transglycosylases have been deleted,[17] has 10-fold increased sensitivity to the cephalosporins cefotaxime, cefamandole, and ceftriaxone because of a permeability defect relative to wild-type MTB.[18] We were able to isolate cefotaxime-resistant mutants of H37RvΔ*rpf*, whereas all attempts to do so for wild-type H37Rv failed.

Resistant mutants were isolated from a population of logarithmically growing H37RvΔ*rpf* at 10 μg/mL cefotaxime (10 times the IC_{90} for this strain); frequency of resistance was $\sim 10^{-7}$. Seven independent clones were isolated showing increased cefotaxime resistance; their precise levels of resistance were measured by determining the concentration of cefotaxime at which 90% of growth was inhibited over five generations of growth in a no-drug control. The clones showed a range of IC_{90} values from 2 to 8 times that of the parent strain (Table 1). Whole-genome sequencing was performed in two of them to identify mutations. Both strains contained single-nucleotide polymorphisms in the gene *ponA2* (Figure 1), which encodes one of two bifunctional PBPs in MTB, so called because they encode both a peptidoglycan glycosyltransferase domain for glycan chain polymerization and a transpeptidase domain for peptide cross-linking of glycan chains.[19] No other mutations were identified in the cefotaxime-resistant strains relative to the H37RvΔ*rpf* parent. The *ponA2* gene in the five remaining resistant strains was sequenced, and one single-nucleotide *ponA2* polymorphism was identified in each strain (Figure 1).

Table 1. IC_{90} Values of H37Rv and Daughter Strains to Cephalosporins

background	*ponA2* allele	fold sensitization relative to wild-type *ponA2*[a]
H37RvΔ*rpf*	parent	1[b]
	ponA2 R81G	4
	ponA2 A96V	2
	ponA2 D112E	4
	ponA2 A150V	2−4
	ponA2 W443S	8
	ponA2 C444S	2−4
	ponA2 A96V *attB::ponA2* WT	1
	ponA2 C444S *attB::ponA2* WT	0.5−1
H37RvΔ*ponA2*	*attB::ponA2* WT	1[b]
	null	32
	attB::ponA2 A96V	1
	attB::ponA2 C444S	4
	attB::ponA2 E100Q (TG-null)	16
	attB::ponA2 S398A (TP-null)	16

[a]IC_{90} values are expressed in multiples of the parent strain resistance. Values are the median measurement from at least four pooled experimental duplicates; a range is reported when an even number of values resulted in a median that was the mean of two median values. The IC_{90} for H37RvΔ*rpf* was 1 μM, and the IC_{90} for H37RvΔ*ponA2 attB::ponA2 (wild-type allele)* was 10 μM. [b]This strain containing the wild-type *ponA2* allele was used as the reference comparator strain for the strains below it in the table.

Unexpectedly, the range of mutations spanned both the glycosyltransferase and transpeptidase domains of PonA2;[20] in other species, mutations linked to resistance typically occur only in the transpeptidase domain, which is the site of β-lactam binding and inhibition.[8,19] All of the mutations were non-synonymous single-nucleotide polymorphisms (Figure 1). None mapped to the catalytic residues of either enzymatic domain.[21−23]

Resistance to β-lactams canonically occurs through target site modification, in which alteration of the drug binding site on the protein surface leads to an antibiotic-refractory enzyme. The altered enzyme, thus uninhibited by the drug, leads to a dominant phenotype for the allele that encodes it. On the basis of this model, we cloned one allele, the W443S allele, the first allele we identified, and expressed it episomally in H37RvΔ*rpf* with the anticipation that this expression would result in a dominant, resistant phenotype. However, despite the use of several different heterologous promoters of various strengths, the introduction of $PonA2_{W443S}$ failed to confer detectable resistance to cefotaxime.

The failure of episomal expression of the resistant mutant alleles to confer resistance combined with the surprising occurrence of mutations in the glycosyltransferase domain, distal to the drug target site, led us to speculate that resistance to cefotaxime might be arising through a different mechanism. In support of this hypothesis, we found that expression of the wild-type allele in a single copy at the heterologous *attB* locus under its native promoter in two of the mutant strains, containing alleles encoding $PonA2_{A96V}$ and $PonA2_{C444S}$, resulted in restoration of sensitivity (Table 1). This finding

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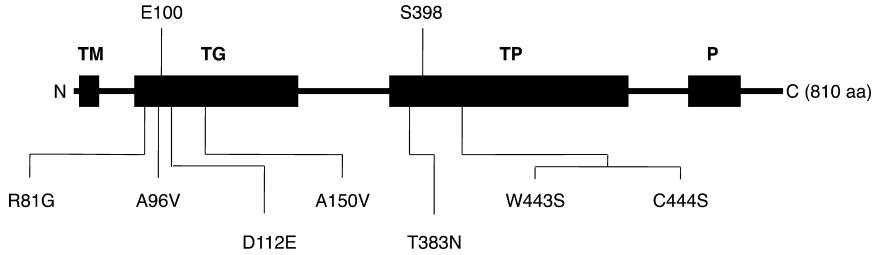

Figure 1. Schematic of PonA2 domain structure and mutations. PonA2 is an 810 amino acid protein containing a transmembrane helix (TM), a glycosyltransferase domain (TG), a transpeptidase domain (TP), and a penicillin-binding protein and serine/threonine kinase-associated domain (P). Above the schematic are indicated the catalytic residues associated with the TG and TP domains. Below the diagram are indicated the amino acid changes resulting from spontaneous mutations conferring cefotaxime resistance.

confirms that these resistance-conferring *ponA2* alleles are indeed recessive.

To determine whether this *ponA2* phenotype was dependent on the H37RvΔ*rpf* strain genetic background, we tested whether the unusual recessive β-lactam resistance mutations could confer resistance in wild-type strain MTB H37Rv. We created a clean deletion of *ponA2* in a wild-type background using the recombineering vector pNit-recET-SacB-kan.[24] Unexpectedly, the complete loss of *ponA2* from H37Rv conferred an even higher level of resistance than the $ponA2_{C444S}$ allele had conferred in H37RvΔ*rpf* (Figure 2A; Table 1).

Complementation of H37RvΔ*ponA2* with $ponA2_{WT}$ under the control of its native promoter suppressed the new resistance phenotype, restoring the wild-type sensitivity to cefotaxime (Table 1). Complementation of H37RvΔ*ponA2* with two of the cefotaxime-resistant alleles A96V and C444S resulted in intermediate phenotypes in which the mutants were resistant relative to wild-type H37Rv, but were less resistant than the strain in which *ponA2* had been deleted entirely (Table 1). The intermediate phenotype of $ponA2_{C444S}$ between $ponA2_{null}$ and $ponA2_{WT}$ led us to the hypothesis that the *ponA2* SNPs we isolated may confer resistance in proportion to the degree to which they have lost protein function, with maximal loss of function resulting in the resistance displayed by Δ*ponA2*; single amino acid substitutions have been shown to cause significant functional impairment in other PBPs.[25] Resistance through loss of function would be consistent with the recessive behavior observed for these mutations.

Previously, it has been shown that disruption of *ponA2* by transposon mutagenesis confers hypersensitivity to heat stress,[26] providing a functional test of PonA2 activity. To determine whether the mutant alleles we had isolated that confer cefotaxime resistance result in partial loss of PonA2 function, their sensitivities to heat stress were tested. The $ponA2_{WT}$, $ponA2_{A96V}$, and $ponA2_{C444S}$ alleles were expressed under the control of their native promoter in H37RvΔ*ponA2*. The mutant alleles conferred significantly increased sensitivity to 24 h of incubation at 45 °C compared to the wild-type allele, as assessed by colony-forming unit (CFU) enumeration (Figure 3). The phenotype suggests that these mutations do in fact cause loss of PonA2 function.

PonA2, like other class A PBPs, has both glycosyltransferase and transpeptidase activities.[19] In addition, the other class A PBP in MTB, PonA1, not only has both of these catalytic activities[19] but also plays a structural role, physically interacting with and contributing to the activity of the peptidoglycan endopeptidase RipA.[27] To determine if loss of one of the catalytic activities was responsible for the observed H37RvΔ*ponA2* phenotype, we mutated a canonical serine residue at position 398 to alanine in the transpeptidase domain. This residue serves as the active hydroxyl nucleophile responsible for forming an acyl-enzyme intermediate in PonA2-mediated transpeptidation.[19] The S398A mutant allele, expressed under the control of its native promoter at the *attB* locus, has substantially higher resistance to cefotaxime than the wild-type allele expressed in H37RvΔ*ponA2*, indicating that loss of the transpeptidase activity alone is sufficient for cefotaxime resistance (Table 1); the expression of the transpeptidase mutant and wild-type alleles is not statistically different, as assessed by qRT-PCR (Figure S1). Furthermore, the transpeptidase mutant shows the same heat stress hypersensitivity phenotype that the $ponA2_{null}$ and *ponA2* single-nucleotide polymorphic alleles showed, indicating that PonA2 transpeptidation is essential for heat stress tolerance as well (Figure 3).

The ability of *ponA2* loss of function to confer cefotaxime resistance suggests that PonA2 is unlikely to be the molecular target of cefotaxime in the classical sense, as would be predicted if resistance had instead occurred by target site modification. Rather, in cases when loss of function leads to resistance, it is often the case that the gene whose function is lost causes a toxic effect in the presence of the drug. This toxic effect may be the enzymatic activation of a prodrug, as in the case of the antibiotics isoniazid and ethionamide, or it may be the accumulation of a toxic intermediate upon inhibition of the targeted enzyme, as in the case of small molecules targeting *glpK*.[28] For β-lactams, a recent model has been proposed that lethality may result from the drug-mediated decoupling of the peptidoglycan glycosyltransferase and transpeptidase activities: the transpeptidase activity, which performs the last cross-linking step, is inhibited by β-lactams, and so un-cross-linked glycan strands from the glycosyltransferase activity accumulate and ultimately lead to cell death through a process under investigation.[29]

If this recently described model is also applicable to MTB, then for PonA2, at least one other transpeptidase must contribute to cross-linking the glycan strands PonA2 produces. This conclusion follows from the fact that we can express the PonA2 transpeptidase-inactivated, glycosyltransferase-intact protein without lethality ($PonA2_{S398A}$; Table 1). In a strain expressing $PonA2_{S398A}$, in the absence of the native PonA2 transpeptidase activity, this putative downstream PBP presumably suppresses downstream toxic effects of PonA2 activity by incorporating products of PonA2 transglycosylation into the sacculus. In this model, genetic inactivation of a downstream PBP would not be predicted to result in β-lactam resistance, as is observed with *ponA2* deletion. Rather, the loss of one downstream PBP would leave diminished detoxifying capacity

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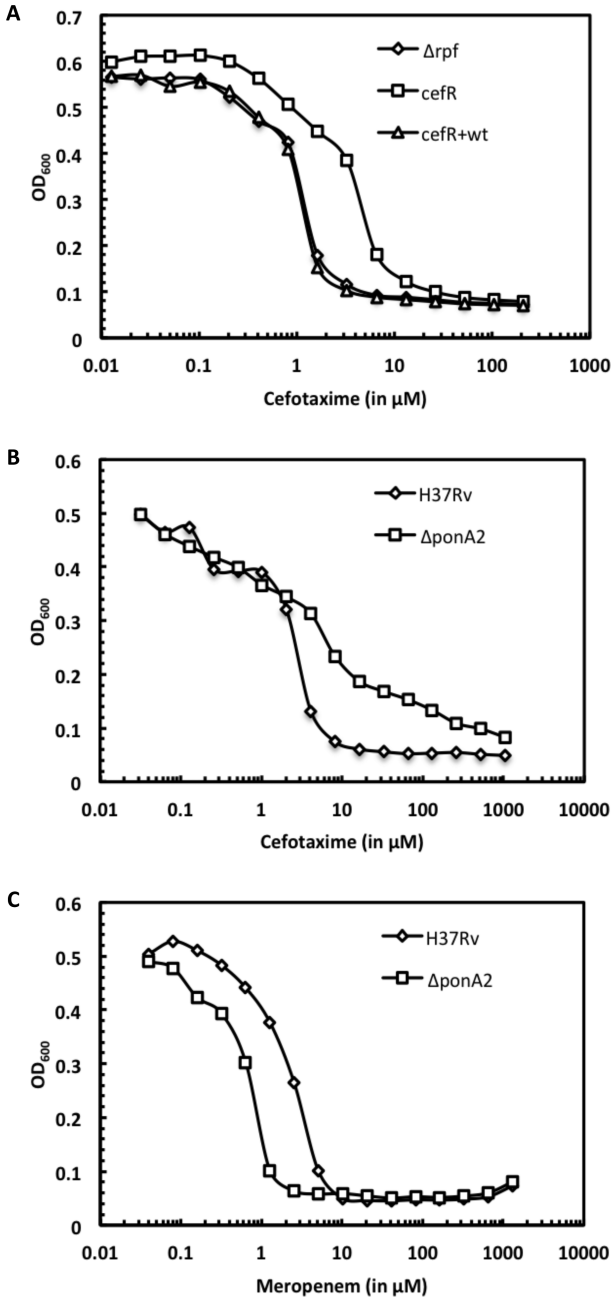

Figure 2. Dose−response curves of MTB and β-lactams. (A) H37RvΔ*rpf* shows resistance to cefotaxime when it contains the allele $ponA2_{C444S}$, but loses resistance when the wild-type allele is coexpressed. (B) Deletion of *ponA2* confers slow-growing resistance at heightened cefotaxime concentrations in wild-type MTB. (C) Deletion of *ponA2* confers sensitivity to meropenem. Assays in each panel were performed together; values charted are the mean of quadruplicates.

in the event of a β-lactam insult, which might be predicted to result in β-lactam hypersensitivity.

Accordingly, we measured the β-lactam susceptibility of strains with disruptions in the remaining predicted PBPs, encoded by *ponA1*, *pbpA*, and Rv2864c. (The final remaining PBP, *pbpB*, is the essential FtsI orthologue and could not be disrupted.) In fact, disruption of each of these PBPs showed no change or modestly increased sensitivity to cefotaxime, cephalexin, and cloxacillin (Figure 4). These findings are

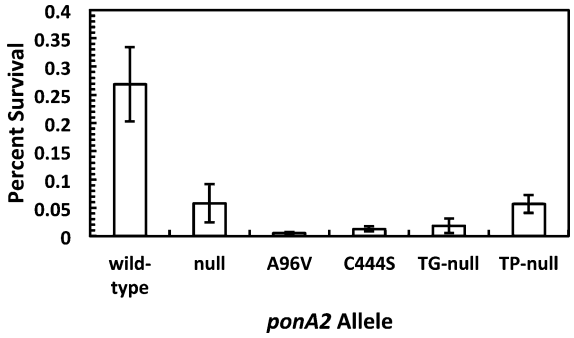

Figure 3. Response of H37Rv mutants containing different *ponA2* alleles expressed from the *attB* locus to heat shock. Cells were heated to 45 °C for 24 h, and their survival was assessed by CFU. The experiment was performed in triplicate. All alleles are significantly different from the wild type at $P < 0.05$, as assessed by Student's two-tailed t test.

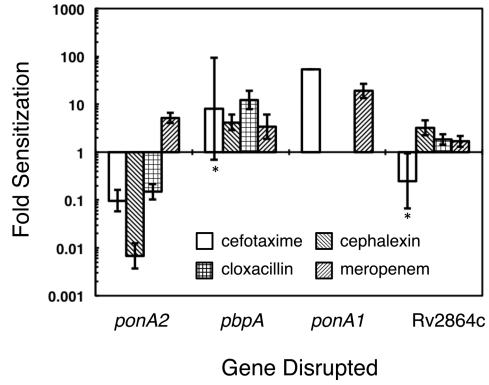

Figure 4. Tabulation of H37Rv mutant susceptibilities to β-lactams. IC_{90} susceptibilities of the indicated PBP mutants were measured by growth of the strains in 2-fold dilutions of the indicated antibiotic for 5 days in 384-well plates. All differences except those indicated by ∗ are significant at $P < 0.05$ as assessed by the Wilcoxon two-tailed rank sum test. Because of the poor stability of cephalexin and cloxacillin in this assay, their susceptibilities were assessed by IC_{50} instead of IC_{90}.

consistent with our proposed model and suggest that any of these three gene products may function downstream of PonA2.

The viability of *ponA2* loss of function mutants suggests that other genes are able to perform peptidoglycan synthesis in lieu of PonA2. We sought to identify these other genes on the basis of the hypothesis that they may become essential when *ponA2* is disrupted but are not essential in wild-type H37Rv in the setting of functional PonA2. We used saturating transposon mutagenesis and massively parallel sequencing (Tn-seq) to perform a negative genetic selection to identify genes essential in H37RvΔ*ponA2* but not wild-type H37Rv. We mutagenized H37RvΔ*ponA2* with the Mariner transposon and collected genomic DNA from the resulting pooled mutants during logarithmic growth[30] to identify genes that are synthetically lethal with *ponA2*. The transposon junctions of the population were then sequenced, and a Bayesian analysis[31] was used to identify candidate genes that are essential in a Δ*ponA2* genetic background but not in the parent wild-type strain, H37Rv. This analysis identified 34 genes required for survival of H37RvΔ*ponA2* but not wild-type H37Rv (Table 2). Of these, 54% are predicted to be secreted or to contain transmembrane helices, as opposed to 26% in the H37Rv genome as a whole.[32] Three of the 34 genes are known extracellular peptidoglycan

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Table 2. Essential Genes in H37RvΔ*ponA2*

gene	transmembrane helix	gene name/function
Rv0016c	yes	*pbpA* (peptidoglycan synthesis)
Rv0049		
Rv0050		*ponA1* (peptidoglycan synthesis)
Rv0067c		transcription factor
Rv0096		PPE1
Rv0129c	yes	*fbpC* (esterase)
Rv0180c	yes	
Rv0238		transcription factor
Rv0431	yes	transcription attenuator
Rv0928		*pstS3* (inorganic phosphate import)
Rv0930	yes	*pstA1* (inorganic phosphate import)
Rv1042c		transposase
Rv1080c		*greA* (transcription elongantion)
Rv1081c	yes	
Rv1157c	secreted	
Rv1158c	secreted	
Rv1340		*rphA* (RNase PH family)
Rv1342c	yes	
Rv1410c	yes	multidrug transporter
Rv1636		universal stress protein
Rv1836c	yes	extracellular solute binding protein
Rv1845c	yes	peptidase
Rv2047c		unknown NADH utilizing phosphotransferase
Rv2138	yes	*lppL*
Rv2198c	yes	*mmpS3* (unknown mycobacterial membrane protein)
Rv2207		*cobT* (B12 biosynthesis)
Rv2224c	yes	unknown hydrolase
Rv2467		*pepN* (unknown aminopeptidase)
Rv2518c	yes	L,D-transpeptidase (putative peptidoglycan synthesis)
Rv2986c		*hupB* (histone-like)
Rv3052c		*ndrI* (nucleotide synthesis)
Rv3645	yes	two-component kinase/signaling
Rv3804c	yes	*fbpA* (esterase)
Rv3810	yes	*pirG* (exported repetitive protein)

transpeptidases: *pbpA*, *ponA1*, and Rv2518c (*ldtMt2*). Because these genes are not essential in wild-type MTB, they must be collectively responsible for maintaining adequate peptidoglycan synthesis in the absence of *ponA2*. In particular, the identification of these genes as part of a parallel pathway to *ponA2* in this assay as well as performing cross-linking downstream of PonA2 as described above suggests that they may accept un-cross-linked glycan strands from both PonA1 and PonA2 as substrates. The enrichment for secreted gene products among the remaining genes suggests that they may contain cellular mechanisms for sensing cell wall stress or for other unidentified functions in peptidoglycan biosynthesis.

Of the three extracellular transpeptidases that we identified, *ldtMt2*, an L,D-transpeptidase, likely plays an important role. We conclude this on the basis of the fact that whereas H37RvΔ*ponA2* shows resistance to cefotaxime (Figure 2B), this strain shows increased sensitivity to meropenem, a member of the carbapenem subclass of β-lactams, suggesting that in the absence of *ponA2*, a meropenem-sensitive mode of peptidoglycan synthesis becomes essential. Of the three candidate genes, *ldtMt2* has been identified as one of the targets of carbapenems, with the paralog of *ldtMt2*, *ldtMt1*, known to bind carbapenems ∼1000-fold more tightly than cephalosporins.[33−35] As the L,D-transpeptidases can form alternative cross-links in a major fraction of the MTB cell wall,[36] it may be that in the absence of cross-linking of glycan strands created by PonA2, LdtMt2's ability to form alternative cross-links becomes essential to maintain the MTB cell wall. Nonetheless, PbpA is likely also inhibited by carbapenems,[37] and LdtMt2 may only act on peptide substrates that have already been cross-linked and/or deanalylated; therefore, the exact roles played by the three transpeptidases identified remain to be investigated.

In conclusion, we report the in vitro generation of β-lactam-resistant mutants in MTB with the surprising finding that loss of *ponA2* function confers cephalosporin resistance. This result strongly supports recent models in which β-lactam killing occurs through an active mechanism, rather than a passive mechanism involving inhibition of an essential PBP. This active mechanism in MTB depends on PonA2, and specifically its transpeptidase domain, because we show that a mutant lacking its transpeptidase catalytic activity, $ponA2_{S398A}$, is relatively resistant to killing by cefotaxime. In contrast to deletion of *ponA2*, deletion of the other PBPs, *ponA1*, *pbpA*, and Rv2864c, results in increased sensitivity to cefotaxime, suggesting that these may be candidate downstream enzymes that play a role in detoxifying some toxic effect of PonA2 glycosyltransferase activity. Furthermore, the viability of PonA2 loss of function mutants and their unique increased sensitivity to meropenem suggest that in PonA2's absence, an alternative meropenem-sensitive peptidoglycan synthetic pathway operates, most likely involving *ldtMt2*, a known target of meropenem. Although the complexity of peptidoglycan biosynthesis and its inhibition by β-lactams leaves many questions to be answered, we believe these findings reinforce with new detail an emerging picture of how multiple PBPs are required to cooperate to build the sacculus and how their inhibition by β-lactams leads to cell death.

MATERIALS AND METHODS

Strains and Growth Conditions. *M. tuberculosis* H37Rv and its derivatives were grown to mid-logarithmic phase at 37 °C in 30 mL sterile square containers; culture aeration was maintained by shaking at 100 rpm with a constant head space of 20 mL. Alternatively, cultures were grown in 1 L cylindrical containers with aeration maintained by rotation at 2 rpm with a head space of >900 mL.

The growth medium was Middlebrook 7H9 Broth supplemented with Middlebrook OADC Enrichment (Becton Dickenson no. 212351), 0.2% glycerol, and 0.05% Tween 80. For solid phase growth, Middlebrook 7H9 Broth was replaced with Middlebrook 7H10 Agar, and 0.5% glycerol was used instead of 0.2% glycerol. For isolation of spontaneous mutants, 25 μg/mL cefotaxime was included in the 7H10 medium.

Genetic Manipulation of *M. tuberculosis*. The *ponA2* mutant alleles were expressed from the *attB* locus using the integrating plasmid vector pMV306-G2, which was derived from pMV306[38] as follows: the small fragment from pNEB193 (New England BioLabs) released by *Eco*RI and *Xba*I was cloned into the *Pac*I site followed by the small fragment from *Sal*I and *Hin*dIII digestion cloned into the *Pme*I site. The resulting construct was digested with *Sgf*I and *Bsp*HI to introduce the zeocin resistance cassette. Alternate alleles of *ponA2* were introduced into this vector using the 800 bp upstream as the native promoter.

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The *ponA2*$_{null}$ strain was generated by one-step double-homologous recombination assisted by the presence of the recombineering vector pNit-recET-SacB-kan, a generous gift of E. Rubin, which was then removed by selection against the *sacB* locus on 5% sucrose.

Dose−Response Curves. Compounds were stored at −20 °C in dimtheyl sulfoxide (DMSO) or at −80 °C for measurement of H37RvΔ*rpfACBED* strains except alleles D112E and A150V in 7H9 medium for all other strains. The potency of compounds was determined as follows: 384-well plates were filled with 20 μL of 7H9 medium per well; if a compound in a DMSO stock was being tested, 384-well plates were filled with 20 μL of 7H9 medium and 1 or 2% DMSO. Compounds were arranged in the plates in 2-fold dilutions from the highest compound concentration to the lowest.

MTB grown to mid-logarithmic phase (OD_{600} 0.5−1.0) was centrifuged at 58*g* to remove clumps and then diluted in fresh 7H9 medium to OD_{600} 0.1. Cells were added in 20 μL aliquots to each well, with individual strains being measured in at least four duplicate wells.

Cells were grown until no-drug OD_{600} reached 0.5−1.0 at 37 °C in sealed containers. After incubation, growth was measured by OD_{600}. The IC_{90} was identified for each dilution series as the lowest concentration at which OD_{600} was <10% of the growth in the absence of drug; the IC_{50} was identified in similar fashion. The strain IC_{90} was reported as the median of duplicates, and a range was reported if the two median duplicates were not identical. In addition to these technical duplicates, all strain and drug combinations were tested in at least two independent experiments. Although absolute numbers differed between experiments due to variation in drug stocks, differences between strains remained constant. In rare cases when the growth in edge wells was significantly below the growth in other wells, growth was normalized to the maximum noninhibitory OD_{600} for a strain rather than to the no-drug control; such cases were selected empirically by cases in which the no-drug control had <50% of the growth of comparable drug wells.

When dose−response curves were measured with an inducible promoter, strains were first grown to OD_{600} 0.5−1.0, diluted to OD_{600} 0.025 in fresh medium in the presence of anhydrotetracycline, and incubated for 7 days using normal growth conditions. Afterward, dose−response curves were prepared normally, but medium contained anhydrotetracycline.

Heat Stress Assay. Cells were pelleted at 2850*g* and washed in 7H9 medium twice. They were then resuspended to OD_{600} 0.05. Cell densities at time zero were assessed by CFU plating. Cells were diluted 1/10 into fresh 7H9 medium and incubated at 45 °C for 24 h. Cell densities were again assessed by CFU plating, with a limit of detection of 10 CFU/mL. All CFU measurements were performed in triplicate, and results are representative of two independent experiments. Heat stress survival was calculated as a percentage of CFU in the culture at time zero.

Transposon Sequencing. A library of 60000 mutants was generated in the H37RvΔ*ponA2* strain on 7H10 agar as previously described. Genomic DNA was isolated from each library and randomly fragmented to 400−600 bp pieces by sonication. Column-purified DNA was split into eight aliquots, which were processed in parallel prior to pooling for Illumina sequencing. Nicked ends were repaired (NEBNext End Repair Module), and A-tails were added with Taq polymerase to allow the ligation of T-tailed adapters (adapter 1.2, TACCACGACCA-NH_2; adapter 2.2, ATGATGGCCGGTGGATTTGTGNNANNANNNTGGTCGTGGTAT; adapters were co-incubated in 2 mM $MgCl_2$ at 95 °C for 10 min followed by slow cooling to 20 °C over a 2 h to allow annealing prior to use). Transposon junctions were amplified for 20 cycles (94 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s) using a primer recognizing the transposon end (5′-TAATACGACTCACTATAGGGTCTAGAG-3′) and one recognizing the adapter (5′-TATGATGGGCGGTGGATTTGTG-3′). The products of these reactions were purified using magnetic beads (Agencourt AmpPure XP) and then amplified for an additional six cycles in a heminested reaction using indexed primers that contained all of the requisite sequence for binding to the Illumina sequencing platform. These products were again purified using magnetic beads and sequenced using the Illumina MiSeq platform.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00119.

Quantitative RT-PCR of *ponA2* alleles expressed from the *attB* locus (PDF)

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Notes

The authors declare no competing financial interest.

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